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Interaction of Human Cytomegalovirus pUL84 with Casein Kinase 2 Is Required for oriLyt-Dependent DNA Replication▽

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Human cytomegalovirus pUL84 is a phosphorylated protein that is required for lytic DNA replication and participates in regulation of virus gene expression. We previously used a proteomics assay to show that human cytomegalovirus pUL84 interacts with casein kinase 2 (CK2). We now have demonstrated that pUL84 is a substrate for CK2 in vitro, and we have determined that two putative CK2 phosphorylation sites within pUL84 mediate binding to CK2. Mutation of a threonine residue at amino acid (aa) 148 and a serine residue at aa 157 within the pUL84 protein resulted in the inability of the protein to interact with the CK2α subunit in transfected cells. Interaction of pUL84 with CK2 was essential for complementation of oriLyt-dependent DNA replication, suggesting that phosphorylation is an essential modification.

Human cytomegalovirus (HCMV) viral DNA synthesis requires both the origin of lytic-phase DNA replication (oriLyt), which is the *cis*-acting replicator (8, 9, 10, 11), and a set of *trans*-acting viral proteins. Initially, 11 distinct loci were demonstrated to be required for oriLyt-dependent DNA replication of HCMV (10, 11). Six of these loci contain homologues or probable homologues of herpes simplex virus type 1 replication genes encoding a DNA polymerase (UL54), a polymerase accessory protein (UL44), a single-stranded DNA-binding protein (UL57), a primase (UL70), a helicase (UL105), and a primase-associated factor (UL102). The other genes required for origin-dependent DNA replication in human fibroblasts (HFs) are the UL84, UL36-38, immediate-early (IE) protein IE1 and IE2, and UL112-113 genes.

pUL84 is a multifunctional protein that plays a role in DNA replication and regulation of gene expression (9). Based on previous findings, pUL84 interacts with oriLyt, most likely within the region of oriLyt that contains RNA/DNA hybrid and stem-loop structures (2). A possible mechanism of initiation of HCMV DNA replication is via direct interaction between pUL84 and specific structures within oriLyt (2).

pUL84 can interact directly with the HCMV regulatory protein IE2 (12), the major transcription-activating protein required for viral gene expression (9). The IE2 protein plays a major role in activating HCMV early promoters and repressing the major IE promoter (7). pUL84, like many regulatory proteins, is phosphorylated; however, the implication of this modification has not been defined (4). In many cases, phosphorylation can dictate protein-protein interactions and nucleic acid binding. Hence, it is important to define the role of phosphorylation in regard to the regulation of protein activity.

Recently we used proteomic analysis techniques to elucidate the pUL84 interactome in infected cells (5). pUL84 binding partners were coimmunoprecipitated from HCMV-infected cells to demonstrate that pUL84 interacts with several virus- and cell-encoded proteins (5). One of these identified cellular proteins was casein kinase 2α (CK2α). CK2 is a serine/threonine-specific kinase composed of catalytic (α or α′) and regulatory (β) subunits that form the holoenzyme tetramer combinations $\alpha_2\beta_2$,$\alpha'_2\beta_2$, or $\alpha\alpha'\beta_2$ (6). This finding suggested that pUL84 could be a substrate for CK2α-mediated phosphorylation.

Computer analysis revealed that there are seven potential CK2 phosphorylation sites in the pUL84 protein (NetPhosK 1.0 software program). The results presented here confirm that CK2 can phosphorylate pUL84 in vitro, and we demonstrate that the amino acid residues T148 and S157 are required for the interaction of pUL84 and CK2α. In addition, we show that mutation of these CK2 sites results in the inability of pUL84 to complement oriLyt-dependent DNA replication.

pUL84 is a substrate for CK2 in vitro. Analysis of the pUL84 open reading frame (ORF) reveals seven consensus CK2 phosphorylation sites at amino acids 64, 65, 74, 148, 157, 292, and 293. Since CK2 was identified as a binding partner for pUL84 in the proteomics analysis of infected cells, we wanted to confirm that pUL84 could act as a substrate for CK2 in vitro. The purified pUL84 protein was produced using the bacterial expression plasmid pET20b(+), containing the pUL84 ORF with an in-frame FLAG epitope, and isolated using a FLAG affinity column (2). CK2 (40 U) was added to 10 μg of purified pUL84 protein in the presence of 10 mM [γ-^{32}P]ATP. After a 1-h incubation at 30°C, the reaction was stopped by adding loading buffer (Laemmli), followed by heating to 95°C, and the protein sample was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The gel was dried, exposed to a phosphorimager screen, and analyzed using a phosphorimager (GE). Under these conditions, pUL84 was efficiently phosphorylated upon addition of CK2 (Fig. 1, lane 2), whereas in the absence of CK2, no signal was detected (Fig. 1, lane 1). These results demonstrated that pUL84 is a substrate for CK2 in vitro.

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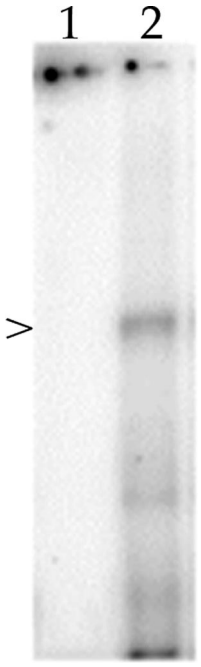

FIG. 1. pUL84 is a substrate for CK2. In vitro phosphorylation assay where purified pUL84 from bacteria was incubated with CK2 in the presence of [γ-^{32}P]ATP for 1 h at 30°C. Samples were resolved through a 10% SDS-PAGE gel, which was subsequently dried, and labeled bands were detected using a phosphorimager. Lanes: 1, pUL84 purified protein plus [γ-^{32}P]ATP; 2, pUL84 purified protein plus [γ-^{32}P]ATP and CK2.

Interaction of CK2 with pUL84. Since data from the in vitro phosphorylation assay coupled with the identification of pUL84 as a binding partner for CK2 strongly suggested that pUL84 is phosphorylated by CK2, we wanted to determine which putative CK2 site(s) encoded by the pUL84 ORF interacted with CK2. To this end, we generated three pUL84 expression plasmids. The first expression plasmid produced a 100-amino-acid peptide fragment from amino acids 1 to 100 encoded by the pUL84 ORF. This peptide includes the putative CK2 phosphorylation sites located at amino acids 64, 65, and 74. The second subclone expressed amino acids 101 to 200 encoded by the pUL84 ORF, which contains the putative CK2 phosphorylation sites located at amino acids 148 and 157. Lastly, a subclone was generated that expressed amino acids 201 to 586 and contained the putative CK2 sites located at amino acids 292 and 293 (Fig. 2A). All subclones were designed such that an in-frame FLAG epitope was present in the expressed proteins. Using these expression plasmids, we performed cotransfection experiments to identify pUL84 protein domains that interacted with CK2 in an effort to identify the region of the pUL84 protein that interacted with CK2. The UL84-FLAG and CK2-hemagglutinin (HA) expression plasmids were cotransfected into 293 HEK cells, and protein lysates were prepared 2 days posttransfection. Protein complexes were immunoprecipitated from these prepared lysates using FLAG affinity beads, followed by elution with 3× FLAG peptide. All pUL84 subclones, including full-length protein, expressed adequate amounts of protein and were easily detectable in protein lysates when anti-FLAG antibody was used for

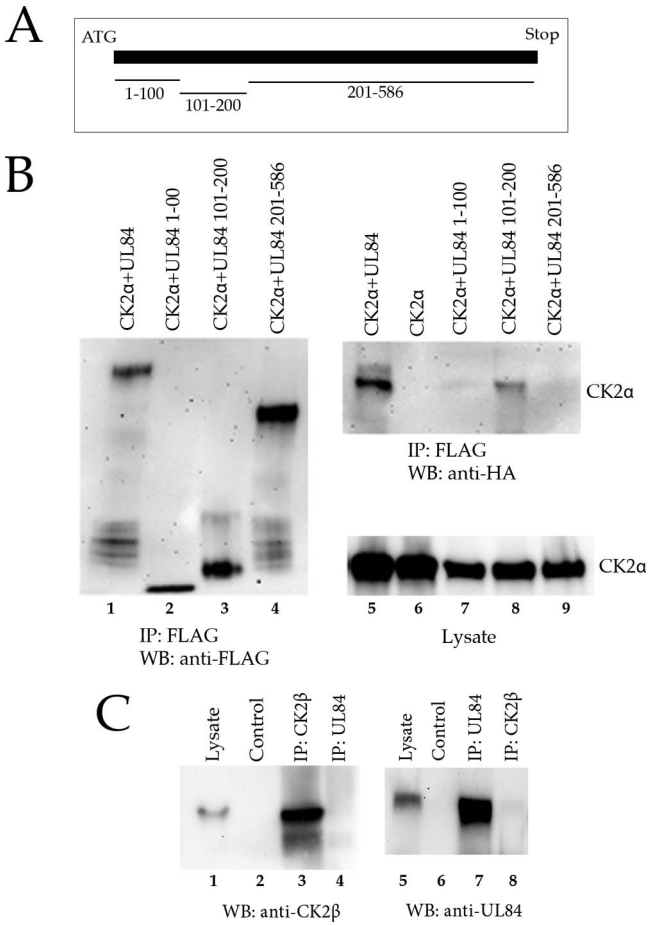

FIG. 2. Localization of the pUL84-CK2 interaction domain. (A) Schematic of the pUL84 ORF showing the location of three subclones that were used in cotransfection immunoprecipitation assays. All subclones were ligated into the expression vector pTARGET and contained an in-reading-frame FLAG tag. (B) pUL84 subfragment encoding amino acids 101 to 200 interacts with CK2. Western blots of cotransfection coimmunoprecipitations from protein lysates using either anti-FLAG (pUL84) or anti-HA (CK2) specific antibodies. Expression plasmids used for the cotransfection mixtures are shown above each lane. Lysate, Western blot of protein lysates from cells cotransfected as above, reacted with anti-HA antibody. (C) pUL84 does not interact with the CK2β subunit. Cotransfection experiments were performed as before except using a CK2β expression plasmid. Expression plasmids used for the cotransfection mixtures are shown above each lane. IP, antibody used for immunoprecipitations; WB, antibody used for Western blot analysis.

immunoprecipitation followed by anti-FLAG Western blot analysis (Fig. 2B, lanes 1 to 4). Coimmunoprecipitations using FLAG affinity beads followed by Western blot analysis of the precipitated protein complexes using anti-HA (CK2) revealed that only the pUL84 fragment encoding the peptide from amino acids 101 to 200 interacted with CK2 (Fig. 2B, lane 8). Also, a Western blot of the protein lysates from cotransfected cells using anti-HA antibody showed the level of CK2 was similar in all transfected samples (Fig. 2B, lysate).

All of the experiments described above were performed using plasmids that expressed the α subunit of CK2. We wanted to investigate if pUL84 interacted with the CK2β subunit. The CK2β subunit does not usually interact with the

substrate for phosphorylation; however, this experiment would serve as a negative control and confirm that pUL84 interacted with only the catalytic subunit of the enzyme. Cells were cotransfected with a plasmid that expressed the Myc-tagged CK2β subunit and the pUL84 expression plasmid. Prepared protein lysates were immunoprecipitated using anti-Myc or anti-pUL84 specific antibodies. Immunoprecipitation experiments failed to show an association between pUL84 and CK2β when either pUL84 specific antibodies or CK2β-Myc antibodies were used (Fig. 2C, lanes 4 and 8, respectively). Control lanes using no antibody failed to show a specific band (Fig. 2C, lanes 2 and 6). This result strongly suggested that pUL84 does not interact with the CK2β subunit.

CK2 binding sites located at amino acid residues 148 and 157 are essential for binding to pUL84. Once it was established that amino acids 101 to 200 encoded by the pUL84 ORF interacted with CK2, we next wanted to determine if the two CK2 consensus sites located within this fragment were responsible for mediating binding. We generated plasmids that expressed mutated forms of the pUL84 ORF. The putative CK2 phosphorylation site located at amino acid 148T was mutated to an alanine residue in the context of the full-length pUL84 ORF (Fig. 3A). We also generated expression plasmids where the CK2 site at amino acid 157S was changed to an alanine residue and an expression plasmid that mutated both putative CK2 phosphorylation sites (Fig. 3A). All pUL84 expression plasmids expressed protein with the FLAG tag. Each of these plasmids was transfected into HEK 293 cells along with the CK2-HA expression plasmid, and coimmunoprecipitations were performed as described above. The pUL84 protein that had the mutated amino acid 148 or 157 still interacted with CK2 in cotransfection immunoprecipitation assays (Fig. 3B, lanes 3 and 4, bottom panel). However, the double-mutant UL84 expression plasmid failed to interact with CK2 (Fig. 3B, lane 7, bottom panel). These experiments strongly suggest that pUL84 is a substrate for CK2 and the interacting amino acids are located at 148 and 157 in the product of the UL84 ORF.

pUL84-CK2 interaction is essential for complementation of oriLyt-dependent DNA replication. Once the amino acids responsible for the interaction with CK2 encoded within the pUL84 ORF were elucidated, we next wanted to investigate if a CK2-pUL84 interaction was required for the replication activity of pUL84. The UL84 expression plasmid UL84-148A/157A, which encoded the mutated version of UL84, was used in the transient cotransfection-replication assay. This assay involves the cotransfection of all of the required replication proteins plus HCMV cloned oriLyt (3, 10, 11). Replicated oriLyt is identified by using the restriction enzyme DpnI, which will cleave input/unreplicated DNA. Amplified oriLyt is then detected by Southern blot hybridization using the parent plasmid vector as a probe. HF cells were transfected with the six HCMV core replication proteins, an IE2 expression plasmid, pGEM-oriLyt, and either a UL84-Tag or UL84-148A/157A expression plasmid. Total cellular DNA was harvested 6 days posttransfection and cleaved with EcoRI and DpnI. DNA was resolved using a 0.8% agarose gel, which was subsequently transferred to a nylon membrane (ZetaProbe; Bio-Rad) and hybridized with a ^{32}P-labeled pGEM7zf(−) vector. The double-mutant pUL84 expression plasmid was unable to complement transient DNA replication, whereas wild-type pUL84

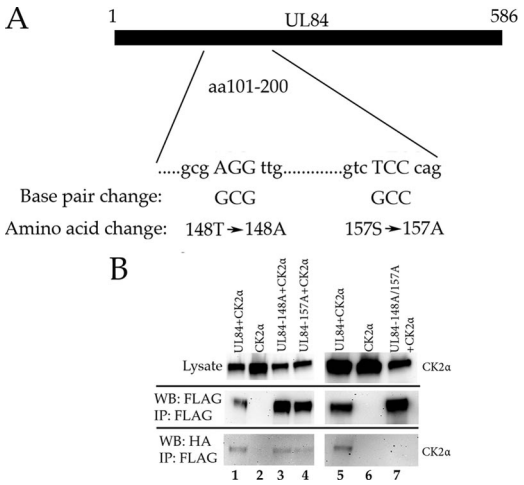

FIG. 3. The putative CK2 phosphorylation sites at amino acid residues 148 and 157 mediate binding with CK2. (A) Schematic of the pUL84 ORF showing the base pair changes incorporated and the resulting amino acid residue changes. (B) Western blot of protein lysate from cotransfection/coimmunoprecipitation assays. The expression plasmids used for cotransfection experiments are shown above the gel lanes. IP, antibody used for immunoprecipitations; WB, antibody used for Western blot analysis; Lysate, protein lysate before coimmunoprecipitation.

transfection resulted in a replication product for oriLyt (Fig. 4, lanes 1 and 2). The control transfection, where UL84 or UL44 (polymerase accessory protein) was absent from the transfection mixture, also showed no replication signal (Fig. 4, lanes 3 and 4, respectively). The single pUL84 mutants were still capable of complementing oriLyt-dependent DNA replication (data not shown). This result strongly suggests that the binding of CK2 is essential for the activity of pUL84 in the context of the replication assay and the phosphorylated version of pUL84 contributes to the replication function of the protein.

The regulation of HCMV lytic DNA replication is poorly understood; however, it is clear that pUL84 plays a central role in the initiation of DNA synthesis. Previously pUL84 was shown to be a phosphoprotein, and phosphoamino acid mapping suggested that the protein was phosphorylated at serine residues (4). Here we show that at least one of the pUL84 amino acid residues that interact with CK2 is a serine residue. The role of phosphorylation of pUL84 had not been established. The results presented here present the first evidence that suggests that phosphorylation of pUL84 by CK2 contributes to the replication function of pUL84. Although it is likely that pUL84 is phosphorylated at other sites, our initial proteomics screen identified only one kinase, CK2, as a binding partner (5). CK2 was shown to be associated with the HCMV virion and was necessary for efficient transactivation of major IE promoter and IE gene expression (8). It is possible that virion-associated CK2 may have implications with respect to pUL84 phosphorylation given that pUL84 is also a component of the virion (13). One speculation concerning the interaction of pUL84 with CK2 is that the two proteins could be part of a more stable functional complex and this complex could function to facilitate the phosphorylation of other viral or cellular proteins. CK2 phosphorylation has been implicated with respect to other HCMV proteins. HCMV IE2 was phosphory-

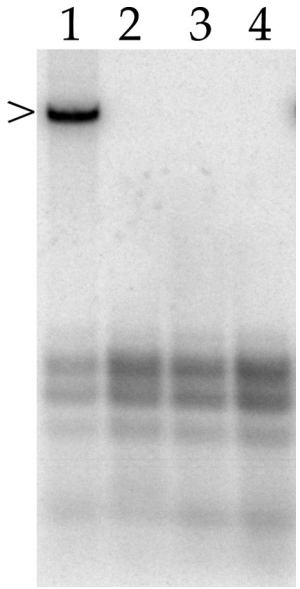

FIG. 4. pUL84 148A/157A mutant fails to complement oriLyt-dependent DNA replication. HF cells were cotransfected with plasmids expressing the required replication proteins and an oriLyt-containing plasmid. Cotransfection mixtures contained either the UL84-Tag or UL84-148A/157A expression plasmid. Total DNA was harvested 6 days posttransfection and cleaved with EcoRI and DpnI, and replicated oriLyt was detected by Southern blotting. Lanes: 1, all required replication proteins along with oriLyt plus UL84-Tag; 2, all required replication proteins along with oriLyt plus UL84-148A/157A; 3, all required replication proteins along with oriLyt, no UL84; 4, all required replication proteins along with oriLyt plus UL84-Tag, no UL44.

lated at consensus CK2 sites (1). The lack of IE2 serine phosphorylation was associated with an increase in transactivation activity of IE2 and in some cases resulted in an overall growth enhancement when mutations were introduced in the IE2 locus in the context of the virus (1). At this time, we do not know the exact function that phosphorylation of pUL84 plays in DNA replication and the activity of pUL84; however, since pUL84 participates in regulation of viral gene expression and DNA synthesis, there is always the possibility that phosphorylation may contribute to several activities.

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